Cdk5 Levels Oscillate during the Neuronal Cell Cycle

Cdh1 UBIQUITINATION TRIGGERS PROTEOSOME-DEPENDENT DEGRADATION DURING S-PHASE*[S]

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Background: Cdk5 is an atypical Cdk that inhibits the cell cycle in post-mitotic neurons.
Results: APC-Cdh1 mediates the degradation of Cdk5 during S phase of the cell cycle.
Conclusion: Maintenance of the proper levels and nuclear location of Cdk5 act to suppress the cell cycle in neuronal cells.
Significance: Subcellular localization of Cdk5 and p35 may play roles in the pathogenesis of Alzheimer disease.

When cell cycle re-activation occurs in post-mitotic neurons it places them at increased risk for death. The cell cycle/cell death association has been reported in many neurodegenerative diseases including Alzheimer disease (AD), yet the mechanisms by which a normal neuron suppresses the cycle remain largely unknown. Recently, our laboratory has shown that Cdk5 (cyclin-dependent kinase 5) is a key player in this protective function. When a neuron is under stress, Cdk5 is transported to the cytoplasm; this eliminates its cell cycle suppression activity and the neuron re-enters S-phase. In the current study we show that a similar principle applies during a normal cell cycle. When a neuronal cell enters S phase, Cdk5 is transported to the cytoplasm where it is ubiquitinated by the E3 ligase APC-Cdh1. Ubiquitinated Cdk5 is then rapidly degraded by the proteasome. The ubiquitination site of Cdk5 appears to be in the p35 binding area; in the presence of high levels of p35, the ubiquitination of Cdk5 was blocked, and the degradation in S phase was attenuated. The data suggest an unsuspected role for Cdk5 during the progression of a normal cell cycle and offer new pharmaceutical targets for regulating neuronal cell cycling and cell death.

The cell division cycle of eukaryotic cells is conventionally divided into four phases: G1, S, G2, and M. Cyclins and cyclin-dependent kinases (Cdks)[3] are two key protein families that help determine the pace of progression through these phases. Cyclins function as the regulatory proteins of the proline-directed serine/threonine kinases. They are up- or down-regulated, depending on the phase of the cell cycle, by both transcriptional and post-transcriptional mechanisms. In a well-coordinated cell cycle, the timely degradation of cyclin proteins is as important

as their synthesis. This degradation is usually mediated by a highly specific ubiquitin-dependent proteolysis (1).

The mammalian Cdk family consists of 10 members: Cdk1 to Cdk9 and Cdk11 (2). Among these Cdks, Cdk5 is regarded as non-traditional. Though its substrate preferences are typical for a Cdk kinase, its activity relies on two specific activator proteins, p35 and p39, not traditional cyclins. These two proteins are structurally similar to cyclins, but share no homology at the amino acid level. Cdk5 is found in many cell types (3, 4); however its activity is primarily detected in the nervous system (5–7) where the levels of p35 and p39 are highest. In addition to the brain, low levels of Cdk5 kinase activity are also present in the adult mouse prostate and embryonic limb buds (8). The function of Cdk5 has been widely investigated during development where it has a recognized role in the phosphorylation of a variety of cytoskeletal proteins (9, 10). In recent years, additional roles for Cdk5 have been discovered in the post-mitotic neuron. Most of these are related to the ability of Cdk5 to phosphorylate numerous synaptic proteins (11–13); there is no reported linkage of Cdk5 activity with the promotion of a normal cell cycle. Despite its apparent outlier status, however, our laboratory has demonstrated that Cdk5 does play a role in cell cycle regulation. It has an unexpected non-catalytic function of inhibiting rather than advancing the cell cycle in post-mitotic neurons (14). Further, this newly discovered activity of Cdk5 appears to be neuroprotective since the unscheduled reactivation of neuronal cell cycle activity is closely linked to neurodegenerative disease (15–17). This linkage and the failure of cell cycle suppression in Cdk5 deficiency has led our laboratory to focus on the biochemistry of neuronal cell cycle regulation in post-mitotic neurons.

We find that Cdk5 normally prevents neuronal cell cycle re-entry by disrupting the E2F1-DP1 complex, inhibiting its access to the promoters of cell cycle genes (19–22). We also find that Cdk5 is a nucleocytoplasmic protein. Its nuclear import depends on its binding the Cdk inhibitor, p27; its nuclear export depends on an endogenous nuclear export signal (NES) and the activity of CRM1. This nucleocytoplasmic shuttling is tightly correlated with the cell cycle. Cdk5 moves to the cytoplasm either during S-phase of a normal cell cycle, or during the DNA replication that occurs when a post-mitotic neuron re-enters

the cell cycle (23). This neuroprotective function, however, contrasts with the finding that Cdk5 hyperactivity, triggered in part by elevated levels of p25 (a breakdown product of p35), is neurotoxic over a timeframe of weeks. Indeed, this toxicity has been proposed as a pathogenic force in neurodegenerative diseases such as Alzheimer disease (24, 25). Paradoxically, our own short-term studies offered hints that over a timeframe of hours, cytoplasmic Cdk5 is neuroprotective (22, 26). This discrepancy led us to an interest in the fate of Cdk5 once it moved to the neuronal cytoplasm. In the current study we show that cytoplasmic Cdk5 is an unstable protein and that its stability is regulated by ubiquitin-dependent proteasomal degradation. We suggest that the failure of this normal clearance pathway would worsen the consequence of any elevation of Cdk5 kinase activity and, coupled with the loss of neuronal cell cycle suppression, suggests that Cdk5 plays multiple roles in the pathway leading to neurodegenerative disease.

EXPERIMENTAL PROCEDURES

Antibodies and Chemical Regents—Antibodies against cyclin A, p27, Cdk3, Cdk4, Cdk6, GFP(B2), GFP(FL), p35(C19), and anti-HA were from Santa Cruz Biotechnology (Santa Cruz, CA). Rat-anti-BrdU, Cdk1, Cdk2, α-tubulin, and ubiquitin antibodies were purchased from Abcam (Cambridge, UK). Secondary antibodies used for immunocytochemistry were as follows: goat anti-mouse Alexa 488 and 594; goat anti-rat Alexa 488 and 594; goat anti-rabbit Alexa 488 and 594 (Invitrogen, Eugene, OR). All were used at a dilution of 1:1000. DAPI ($4',6'$-diamidino-2-phenylindol) was used as a nuclear counterstain at 1 μg/ml. MG132, cycloheximide, and nocodazole were from Sigma. DynBeads was from Invitrogen, Inc. β-amyloid1–42 was purchased from American Peptide Company (Sunnyvale, CA), β-amyloid1–42 was aged 5 days at 37 °C before use.

DNA and Constructs—GFP-cdk5, GFP-p35, HA-cdk1–9, Cdh1, Cdc20, and Skp2 were purchased from Addgene Inc. GFP-p10 was constructed by inserting a sequence encoding the p10 fragment of Cdk5 in EGFP-C3. All truncated Cdk5 constructs were made as described previously (22).

Cell Culture and Synchronization—N2a (Neuroblastoma 2A) cells were purchased from ATCC. N2a cells were cultured in DMEM media supplemented with 10% FBS. For cell synchronization, 50 ng/ml nocodazole were added to the media for 18 h, following which the cells were washed with fresh media and cultured in normal DMEM. Cells were harvested at different times following removal of nocodazole from the medium.

Primary Neuronal Cultures—Embryonic cortical neurons were isolated by standard procedures. Isolated C57BL/6J E15.5 embryonic cerebral cortices were treated with 0.25% Trypsin-EDTA and dissociated into single cells by gentle trituration. Cells were suspended in Neurobasal medium supplemented with B27 and 2 mM glutamine, then plated on coverslips coated with poly-L-lysine (0.05 mg/ml). All cultures were grown for a minimum of 5 days *in vitro* (DIV) before any treatment. To assess cell cycle activity, medium was exchanged with fresh medium containing 10 μM BrdU. After 12 h, cultures were fixed with 4% paraformaldehyde, then washed and stored in PBS. All experiments were performed on a minimum of three litters; each condition was examined in triplicate.

Immunocytochemistry and BrdU Incorporation—At the appropriate time, cultures were rinsed once with PBS and then exposed to 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at room temperature followed by three rinses with PBS. Immunocytochemistry of cell cultures was done without antigen retrieval. For BrdU labeling, the cells were serum starved for 48 h followed by 12 h of serum add-back. Four hours before the end of the experiment, 10 μM BrdU was added to the media. The cells were then fixed, and DNA was hydrolyzed by exposing the cells to 2 N HCl for 10 min. Specimens were neutralized in 0.1 M sodium borate (pH 8.6) for 10 min, then rinsed extensively in PBS (3×) for 45 min before treatment with blocking reagent. Nonspecific antibody binding was blocked by exposing the fixed cells to 5% normal goat serum in 0.1% Triton X-100 for 1 h before application of the primary antibody.

Western Blotting and Co-immunoprecipitation—Dissected tissues or harvested cells were homogenized in 1:5 (w:v) ice cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl), plus protease inhibitor mix (Roche, Basel, Switzerland). The samples were centrifuged at 12,000 × g for 20 min at 4 °C. The supernatant was collected, and total protein levels were measured by a Micro Bicinchoninic Acid (BCA) protein assay kit (Pierce Biotechnology). For Western blots, lysates were separated with SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBST and probed with primary antibodies in blocking buffer, followed by treatment with HRP-linked secondary antibodies and ECL Western blotting detection reagents (Pierce Fisher Scientific). For immunoprecipitation, the protein lysates were first cleaned by incubation with Protein G beads for 30 min at 4 °C, and then the desired antibody was used to precipitate the antigen overnight at 4 °C. After washing with IP buffer, the immunoprecipitated beads were boiled in loading buffer for Western blotting experiments. The intensity of immunoreactive bands was quantified using NIH Image.

Flow Cytometry Assay—N2a cells were harvested and washed by PBS. 3 ml of ice-cold 70% ethanol was slowly added, dropwise, while vortexing the cells. The suspension was then placed on ice for 30 min, after which the cells were lightly centrifuged (300 × g) for 5 min. The supernatant was then aspirated, and the cells washed with 3 ml of PBS. After ethanol fixation, the cell pellets are loose so extra care was taken at this step not to lose any cells. The cell pellet was resuspended in 500 μl of PBS; 50 μl of RNase A solution and 5 μl of PI (1 mg/ml) were added, and the suspension incubated at room temperature for 30 min in the dark. The cells were then analyzed by flow cytometry on a Beckman Coulter FC500 Analyzer.

RESULTS

Cdk5 Levels Drop during S Phase—We synchronized N2a cells in G2/M-phase by exposing them to nocodazole for 18 h in culture. After release of the cell cycle by shifting to nocodazole-free medium, cells were harvested at various times for assay. Each sample for each time point was split in two. One aliquot was used for flow cytometry to assess the stage of the cell cycle (Fig. 1*A*); the second was used for Western blots to determine the levels of various proteins (Fig. 1*B*). After release, N2a cells passed from M phase into G1 phase; then, at about 13 h, the
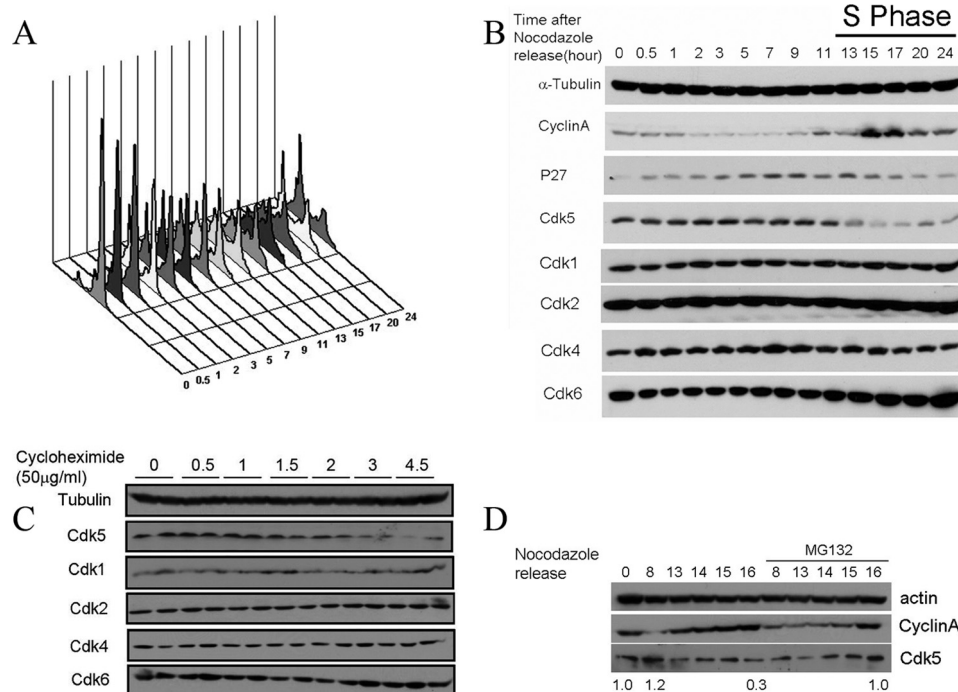
FIGURE 1. **Cdk protein levels during cell cycle.** N2a cells were cultured in DMEM with 10% FBS. Treatment with nocodazole (18 h) was used to synchronize the cell cycle. *A*, cells were harvested at different times after removal of nocodazole followed by analysis of cell cycle phase by flow cytometry. *B*, aliquots of cells analyzed were used to detect the levels of various cell cycle proteins as indicated. Tubulin served as loading control. *C*, cycloheximide was used to inhibit protein synthesis. Subsequently, the stabilities of the Cdks were assessed by Western blotting at the times indicated. *D*, MG132 was used to block proteasome activity. N2a cells were synchronized by nocodazole in the presence of MG132; the remaining levels of Cdk5 and cyclin A were measured by Western blot. Actin served as a loading control.

cells entered S phase This is best seen by noting the levels of the S-phase cyclin, Cyclin A (Fig. 1*B*, *row 2*). The levels of the Cdk inhibitor (CKI), p27, mark the G1 phase of the cycle (Fig. 1*B*, *row 3*).

The protein levels of typical Cdks (Cdk1, Cdk2, Cdk4), remain constant during a cell cycle (Fig. 1*B*, *rows 5–7*). Two curiosities appear in these data, however, the increases of Cdk6 11 h after nocodazole release (Fig. 1*B*, *rows 8*). It may be that the increased activity of Cdk6 required to promote the cell cycle is achieved in part by an increase in protein levels. Given that it is not normally thought of as participating in the cell cycle, it is unexpected that the Cdk5 protein was substantially down-regulated during S phase 13 h after nocodazole release (Fig. 1*B*, *row 4*).

Cdk5 Is an Unstable Protein—We deemed it unlikely that the levels of Cdk5 were regulated by transcription; rather, our data suggest that Cdk5 is an unstable protein whose degradation is enhanced during S-phase of the cell cycle. To investigate this possibility, cycloheximide was used to block new protein synthesis. In this situation, the levels of a protein detected on a Western blot reflect only its rate of degradation. When 5 different Cdk proteins were analyzed in this fashion, only the levels of Cdk5 dropped significantly (Fig. 1*C*). Quantification of the gels revealed that the Cdk5 half-life is about 2 h under these conditions. Other Cdks such as Cdk1, 2, 4, and 6 were not detectably degraded during the entire 4.5 h of the experiment. To determine whether the loss of Cdk5 during S-phase was regulated by the proteasome, we used MG132 to inhibit proteasomal activity after nocodazole release. As shown in Fig. 1*D*, the down-regulation of Cdk5 in S phase shown in Fig. 1*B* was blocked by

MG132. In untreated cultures, the Cdk5/actin ratio was reduced to 30% of its initial value by 16 h after nocodazole release by which time the cells were in mid S-phase. In the presence of MG132, however, the Cdk5/actin ratio was unchanged during this time.

The data suggest that a coordinated reduction in the levels of Cdk5 is necessary for a cell to enter S phase. This is consistent with our earlier findings (20–22) and is particularly relevant for neuronal survival as mature CNS neurons are normally non-mitotic; their forced re-entry into a cell cycle will kill them (27). Indeed, neuronal cell cycle reactivation has been widely reported in Alzheimer disease (15, 16, 28–29). As β-amyloid is a potent neurotoxin that is present in the AD brain, and previous reports have shown that it can induce normally post-mitotic neurons to re-enter a cell cycle, we used it to trigger the cell cycle activity of mouse neocortical neurons. As shown in supplemental Fig. S1, β-amyloid administration successfully induced neuronal cell cycle reentry. The endogenous Cdk5 level was tracked during this process by the addition of BrdU 12 h before fixing the cultures. (Fig. 2*A*). We consistently found that the BrdU-positive S-phase neurons (Map2-positive cells) had low overall Cdk5 immunostaining, particularly in the nucleus, while the BrdU negative neurons had strong, predominantly nuclear, Cdk5 staining. Combined with the results from the N2a cells, these data implicate the degradation of Cdk5 as a critical event that precedes neuronal cell cycle activation in both dividing and non-dividing cells.

The Cdk5 stability data presented thus far emphasize the importance of degradation in the regulation of Cdk5 protein levels. This suggests that the developmental up-regulation of
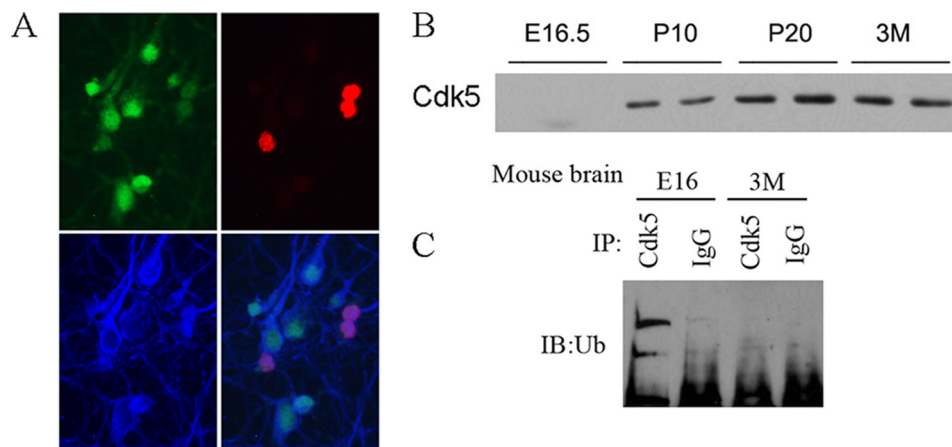
FIGURE 2. **Cdk5 is maintained at low levels in cycling neurons and developing brain.** *A*, dissociated cortical neurons were cultured from E16. 5 mouse embryonic cortex. Neurons were treated with β-amyloid in DIV5. Triple-staining was performed on the fixed neurons: Cdk5 location (*green*), BrdU incorporation (*red*), and the neuronal marker, Map2 (*blue*), were assessed by immunostaining. *B*, nuclear fractionation was performed on lysates of mouse cortex ranging in age from E16 to 3 months. The levels of nuclear Cdk5 were detected by Western blotting. *C*, endogenous ubiquitination of Cdk5 in embryonic brain and adult brain. Whole brain lysates were immunoprecipitated with Cdk5 antibody. The precipitates were run on SDS gels, blotted, and the levels of ubiquitination determined by Western blotting with anti-ubiquitin antibody.
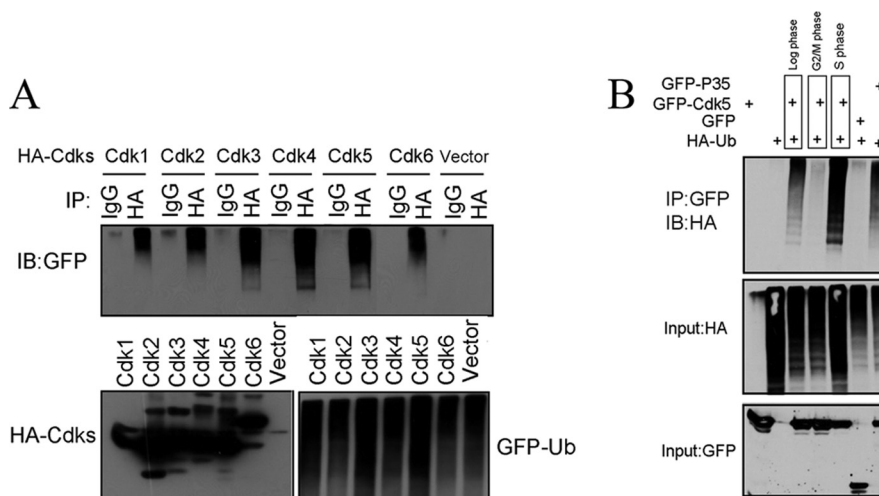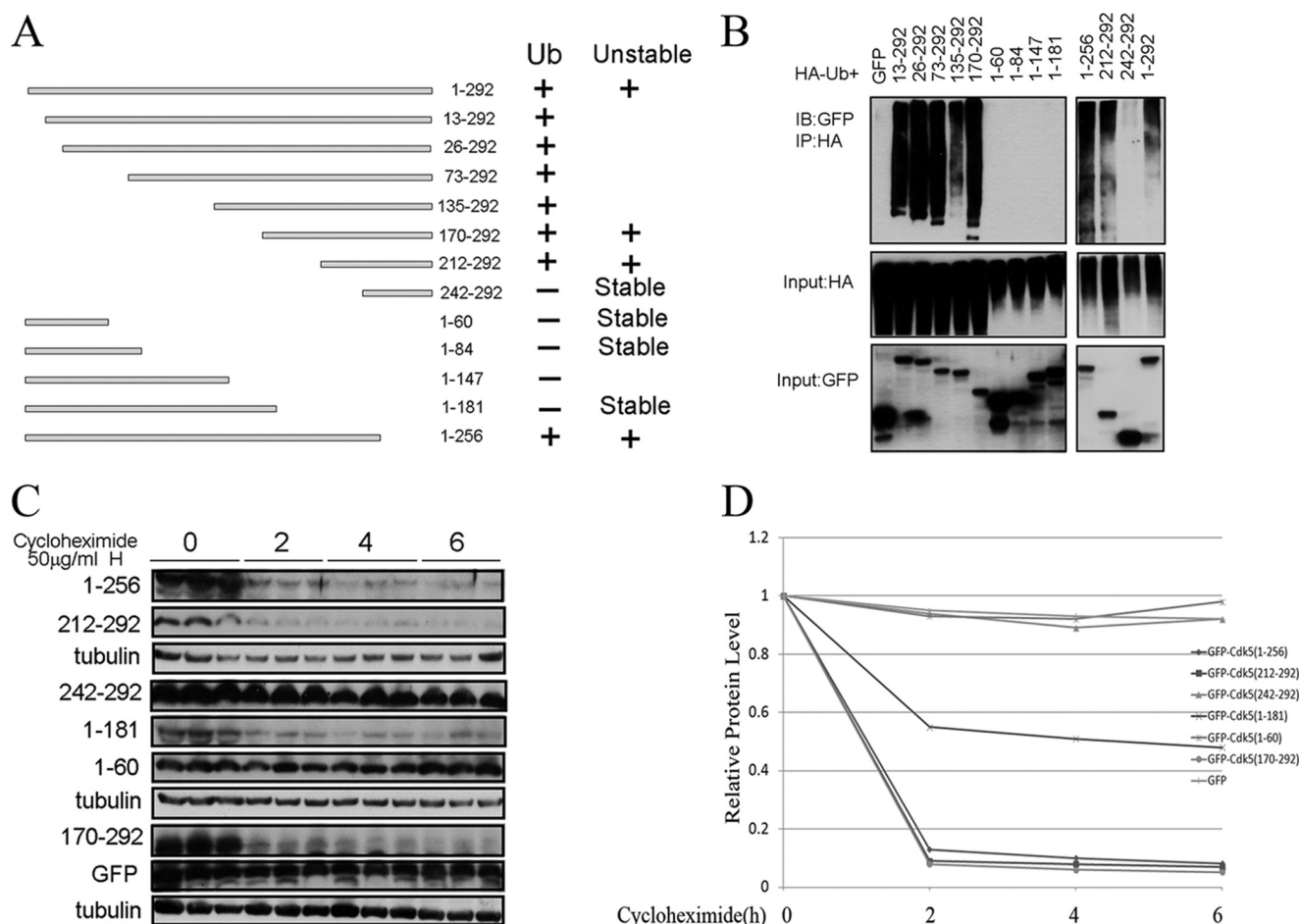

FIGURE 3. **The ubiquitination of Cdks.** *A*, constructs encoding HA-tagged Cdk1–6 and control vector were co-transfected with GFP-ubiquitin (GFP-Ub) into N2a cells. Cell lysates were immunoprecipitated wtih HA, and blotted with GFP to assess the levels of Cdk ubiquitination. The input lysates reveal comparable expression and loading. *B*, ubiquitination of Cdk5 in different cell cycle phases. GFP, GFP-Cdk5, and GFP-p35 (as indicated) were co-expressed in N2a cells with HA-tagged ubiquitin. After transfection, cells were harvested while still in log phase, or at specific times after nocodazole release corresponding to G2/M-phase, or S-phase. Ubiquitination was determined by immunoprecipitation with GFP followed by Western blotting with HA antibody.

Cdk5 (30) might be due to decreased proteasomal degradation. The involvement of the proteasome suggests further that ubiquitination of Cdk5 might be a key regulatory event. To test this idea, we first validated previous reports that during the early stages of normal embryonic brain development, Cdk5 levels are very low but increase substantially as brain maturation proceeds (Fig. 2*B*). Then we examined the levels of Cdk5 ubiquitination in embryonic (E16) and adult (3-month-old) brain. Fig. 2*C* shows that, consistent with our hypothesis, Cdk5 was far more strongly ubiquitinated in the early stages of brain development (E16) compared with the adult brain (3 M), suggesting that in embryonic brain, when cell division is active, Cdk5 is highly ubiquitinated, and its protein level is suppressed.

Cdk5 Is Specifically Ubiquitinated in Cycling Cells—To pursue the role of ubiquitination in Cdk regulation, multiple HA-tagged Cdks were co-transfected with GFP-ubiquitin into log-

phase N2a cells. Cell lysates were immunoprecipitated with HA, run on Western blots and blotted with GFP. Under these conditions, we detected robust ubiquitination of all Cdks tested (Fig. 3*A*). To determine whether the ubiquitination of Cdk5 was correlated with the changes in its levels during the cell cycle, Cdk5, and ubiquitin were co-transfected into N2a cells, which were then exposed to nocodazole to establish a G2/M-phase arrest. The nocodazole block was released and, based on the timing established in Fig. 1*A*, aliquots of cells were harvested in log phase, G2/M phase and S phase. The results show that the ubiquitination of Cdk5 is very low in M phase, but very high during S phase. The p35 protein was used as a positive control to confirm the ubiquitination reaction (Fig. 3*B*).

To investigate where on Cdk5 the ubiquitin moieties were added, we analyzed several constructs encoding variant forms of Cdk5 in which portions of the protein were deleted (Fig. 4*A*).

FIGURE 4. **Mapping of the ubiquitination and degradation pattern of Cdk5.** *A*, a diagrammatic representation of the various Cdk5 truncation constructs, their ability to be ubiquitinated (*Ub*) and their stability. *B*, different truncated GFP-tagged Cdk5 constructs were co-transfected with HA-tagged ubiquitin. After IP with GFP their ubiquitination were detected by Western blotting with HA antibody. *C*, representative truncated Cdk5 mutations were transfected into N2a cells. Cycloheximide treatment was performed to block new protein synthesis, and the stability of the truncated Cdk5 proteins were measured by Western blotting. *D*, protein degradation curves for *panel C*.

These vectors were co-transfected with HA-tagged ubiquitin, and analyzed as described above. Cdk5 isoforms that were missing the C terminus of the protein, for example Cdk5(1–60) and Cdk5(1–181) could not be ubiquitinated (Fig. 4*B*). Deletions of the N terminus, Cdk5(13–292) and Cdk5(170–292) were readily ubiquitinated. This places the site of ubiquitination after amino acid 181. The position was refined further by analysis of two additional constructs. Cdk5(212–292), missing most of the N terminus could not be ubiquitinated while Cdk5(1–256) missing only a portion of the C terminus could be ubiquitinated (Fig. 4, *A* and *B*). This identifies the site as lying somewhere in the 30 amino acids between leucine 212 and tyrosine 242.

The same truncation constructs were then used to validate the role of ubiquitination in regulating Cdk5 stability. We monitored the cellular stability of representative fragments by following their decay in the presence of cycloheximide. In every case, the Cdk5 fragments that our earlier work had determined would be ubiquitinated proved unstable in the absence of new protein synthesis while those that could not be ubiquitinated remained stable up to 6 h (Fig. 4*C*). Fig. 4*D* represents a quantification of these results.

Cdh1 Is the Ubiquitin E3 Ligase that Mediates the Degradation of Cdk5—There are three major ubiquitin E3 ligases that are responsible for cell cycle protein degradation: APC^cdc20, APC^Cdh1, and SCF(skp2). Since Cdk5 was degraded during a specific phase of the cell cycle, we hypothesized that one of these three proteins would mediate its ubiquitination. We transfected expression constructs encoding one of three E3 ligases into N2a cells. Cells were either left untreated or incubated in MG132 to inhibit proteasome activity. In control cells that received no E3 ligase construct (Fig. 5*A*, pCDNA3.1), proteasome inhibition made little difference in the levels of either endogenous Cdk5 or exogenous GFP-Cdk5. When we overexpressed Cdh1, however, Cdk5 protein levels dropped (both exogenous and endogenous) and this drop was prevented by MG132 (Fig. 5, *A* and *C–D*). Neither Cdc20 nor Skp2, by contrast, had any effect on the levels of Cdk5 (Fig. 5, *B* and *C–D*). This identifies Cdh1 as the ubiquitin ligase that is most likely responsible for the cell cycle dependent degradation of Cdk5. To validate this point, we repeated the assay using Cdh1 and several of the Cdk5 fragments assayed above. As shown in Fig. 5, *E* and *F*, co-transfection of Cdh1 with fragments that were able to be ubiquitinated were unstable, but rescued by MG132.
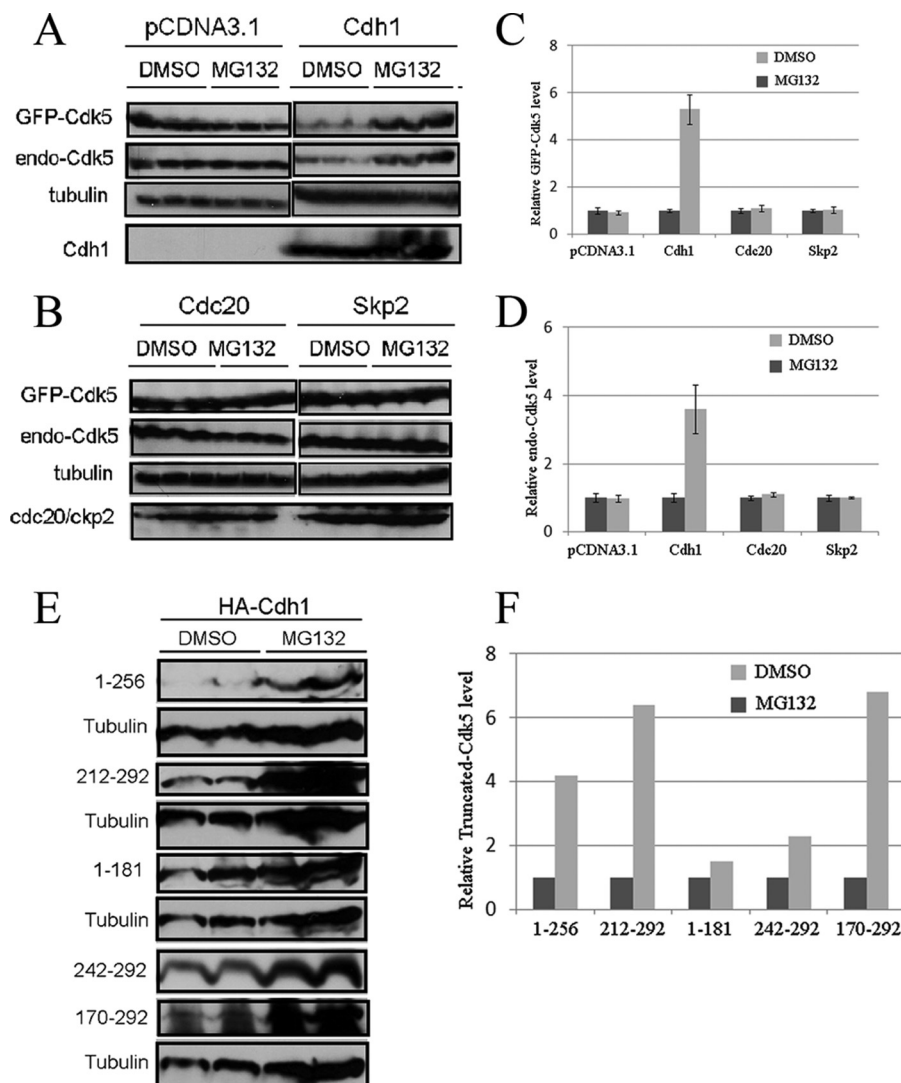
FIGURE 5. **E3 ligase Cdh1 mediated the degradation of Cdk5.** *A–D*, Cdk5 was co-transfected with a single cell cycle-related E3 ligase: Cdh1, Cdc20, Skp2 or an empty pCDNA3. 1 vector. MG132 was used to inhibit proteasome activity (DMSO served as a solute control). If proteasome activity degrades the protein then the protein levels should decline in the DMSO control lanes, but not in the MG132 samples; this is case only for the Cdh1 samples. Level of transfected (GFP-Cdk5) and endogenous Cdk5 (endo-Cdk5) were detected by Western blotting. All experiments were run in triplicate. *E* and *F*, GFP-tagged Cdk5 truncation mutations 1–256 (Cdk5 fragment containing amino acids 1 to 256), 212–292, 181–292, 242–292, and 170–292 were co-transfected with HA-Cdh1 and incubated in the presence or absence of proteasome inhibition (MG132). The effect of Cdh1 on the degradation of these truncations was monitored by Western blot to determine the levels of GFP-tagged protein remaining in the samples.

Those fragments that could not be ubiquitinated were insensitive to MG132 administration.

p35 Blocks the Ubiquitination of Cdk5—Protein ubiquitination requires the binding of the ubiquitin ligase to the target protein. Nuclear Cdk5 exists in a 4-protein complex with p35, E2F1 and p27 (21, 22), and we had previously shown that overexpression of p35 alone was capable of driving complex formation in N2a cells. We wondered, therefore, whether the formation of this complex would affect the ubiquitination of Cdk5 or other Cdks. To test this hypothesis, HA-Cdks were co-transfected with p35 and GFP-Ub. The ubiquitinations of the Cdks were detected as described above. In the presence of p35, the ubiquitination of Cdk1 and Cdk5 was significantly reduced compared with the IgG control (Fig. 6*A*; compare also with Fig. 3*A*). The ubiquitination of other Cdks, such as Cdk2, Cdk3, Cdk4, Cdk6, and Cdk9 were not affected by p35.

Previous work has shown that p35 is an unstable protein; in neurons under stress it can be cleaved into two smaller proteins: p25 and p10. Little is known about the functions, if any, of p10. The p25 fragment, however, has been suggested to stabilize Cdk5 and result in its hyperactivation (24, 31), suggesting that p25 might also be capable of inhibiting Cdk5 ubiquitination. The outcome of this experiment would also serve as a test of whether the larger E2F1/p27/p35 complex was involved since p25 is incapable of driving its formation (21). We used N2a cells to overexpress p25, p10, or ΔP35, a mutant form of p35 that lacks the Cdk5 binding domain. We then repeated the ubiquitination experiment illustrated in Fig. 3*A*. As shown in Fig. 6*B*, p25 can potently inhibit Cdk5 ubiquitination while p10 cannot. The ΔP35, which cannot bind with Cdk5, also cannot suppress the ubiquitination of Cdk5. That Cdh1 is the E3 ligase for the degradation of Cdk5 (Fig. 5) raises the question of whether p25/
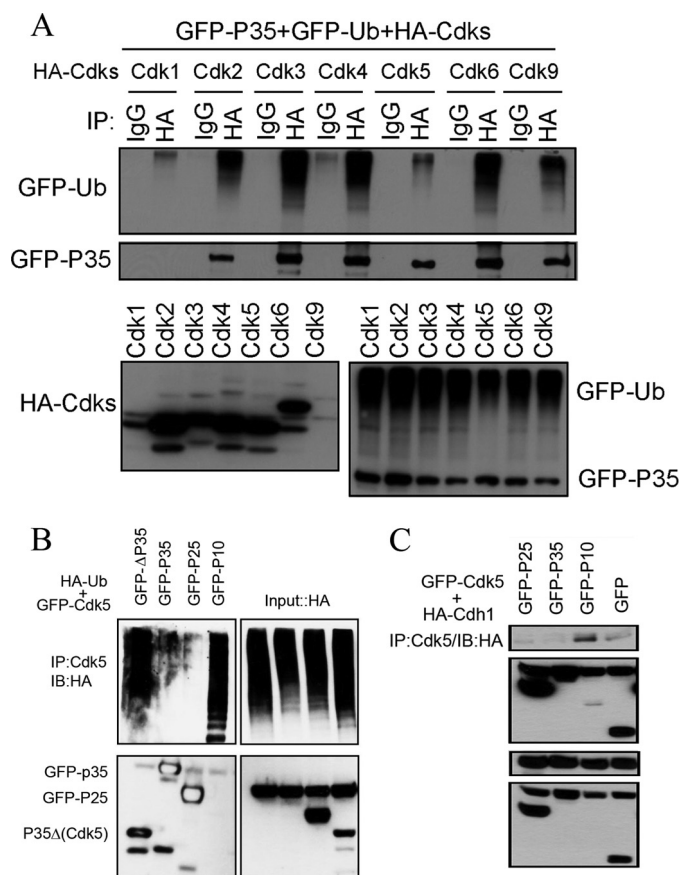
FIGURE 6. p35 blocks the ubiquitination of Cdk5. *A*, GFP-p35 and GFP-Ub were co-transfected with HA-tagged Cdk1–6 and Cdk9 into N2a cells. The Cdks from whole cell lysates were immunoprecipitated with HA or control IgG, followed by Western blotting with GFP to detect any associated p35 or ubiquitin. The expression levels were monitored by Western blotting with antibodies against HA. *B*, GFP-Δp35, GFP-p35, GFP-p25, and GFP-p10 were co-transfected with GFP-Cdk5 and HA-Ub in N2a Cell. Cdk5 was immunoprecipitated by Cdk5(C-8) antibody, and Western blots of the precipitate were probed with HA antibody. (*C*) GFP-p25, GFP-p35, GFP-P10, and GFP control were co-transfected with HA-Cdh1 and GFP-Cdk5. The interaction between Cdk5 and Cdh1 was measured by immunoprecipitation of Cdk5 followed by Western blotting of the precipitate with HA antibody.

p35 can block the interaction between Cdk5 with Cdh1. GFP-Cdk5/HA-cdh1 were overexpressed in the presence of extra p25, p35, p10, or control GFP. As shown in Fig. 6*C*, extra p25, and p35 significantly reduced the physical interaction between Cdk5 and Cdh1.

p35 Attenuates the Degradation of Cdk5—The Cdk5 C-terminal fragment Cdk5(212–292) cannot bind p35 (data not shown), but it can be ubiquitinated (Fig. 4). In keeping with this, we found that Cdk5(212–292) was unstable, and its degradation was not affected by overexpression of either p25 or p35. Overexpression of Δp35, or p39 had no effect on the stability of Cdk5(212–292) (Fig. 7, *A–C*). In contrast, the Cdk5 N-terminal fragment (Cdk5 1–256) can also bind p35 and p25, and it is also ubiquitinated. As predicted, therefore, p25 or p35 can significantly block the degradation of Cdk5(1–256). The degradation of Cdk(1–256) is slower in the presence of p25 than p35, which offers a hint that the shorter Cdk5(1–256) fragment is more effectively stabilized by p25 than by p35 (Fig. 7*B*). Finally, since we showed that Cdk5 was specifically degraded during S-phase of a normal cell cycle, we asked whether additional p35 or p25 also

inhibited this degradation. We overexpressed p25 or p35 in N2a cells and repeated the nocodazole release experiment. The blots show that this manipulation significantly attenuated the degradation of Cdk5 in S phase (Fig. 7, *D* and *E*). At the same time, when we overexpressed the Cdk5(212–292) truncation lacking the p35 binding domain, its degradation during S phase was unaffected, even in the presence of high p35/p25 level (Fig. 7, *D* and *F*).

DISCUSSION

The regulation of all Cdk activities is critical for a cell to properly navigate a cell cycle. During a normal cell cycle, the concentration of the Cdks themselves remains relatively constant. The needed regulation is achieved through dynamic changes in the concentrations of the cyclins and Cdk inhibitors (such as p27 or p16) (32). This picture applies to most of the Cdks we examined during the N2a cell cycle. The levels of Cdk1, Cdk2, and Cdk4 protein all remained constant while the levels of their cyclin partners oscillated substantially (Fig. 1).

The pattern of Cdk constancy during the cell cycle is violated, however, by the Cdk5 kinase whose levels decrease substantially during S-phase of the N2a cell cycle. In an almost cyclin-like pattern its levels follow a near perfect inverse relationship with Cyclin A, an S-phase partner of Cdk2. Fig. 2 shows that this relationship applies not only to cycling neuroblastoma cells, but also to non-cycling primary neurons in culture. In these cells, endogenous Cdk5 and BrdU (an S-phase marker) also show a strong inverse correlation with one another. This is significant as there is considerable data documenting that if such post-mitotic neurons are forced to re-enter a cell cycle they will die rather than divide, as they do in several different neurodegenerative diseases and their mouse models (16–17). Combined with our previous data documenting a cell cycle suppressor function for Cdk5 when it is present in the nucleus (22), we conclude that from the time they are mitotic precursors until their maturation into adult post-mitotic cells and beyond, neurons rely on Cdk5 to meet a life-long need to suppress re-entrance into S-phase.

Unlike most Cdks, but similar to most cyclins, the levels of Cdk5 are mediated primarily by its degradation at a specific moment in the cell cycle. Cyclin degradation during the cell cycle proceeds by ubiquitin-mediated proteolysis (1), and our data show that the same is true for Cdk5. It is particularly intriguing that Cdh1 is the E3 ligase responsible for adding ubiquitin to Cdk5 to target its degradation. The E3 ubiquitin-ligases are responsible for substrate recognition (33), and the two principal E3-ligases involved in cell cycle control are the SCF and APC/C (anaphase-promoting complex/cyclosome) complexes (34). The SCF complex is used throughout the cell cycle whereas APC/C is active from mitosis only through early G1 (35). APC/C is a multiprotein complex that can switch between two major activator proteins, Cdc20 and Cdh1, depending on the cell cycle phase (36–38); Cdh1 is used primarily in early M-phase while Cdc20 functions during late M- and early G1-phase.

Superficially, this offers a rationale for APC[Cdh1] being the enzyme to regulate Cdk5 levels during the N2a cell cycle. But the timing of Cdk5 ubiquitination is out of phase with the reported changes in APC[Cdh1] activity. APC[Cdh1] is usually inactivated prior to initiation of S phase (39), resulting in the stabilization of cyclin A (40). Unexpectedly, our data demonstrates
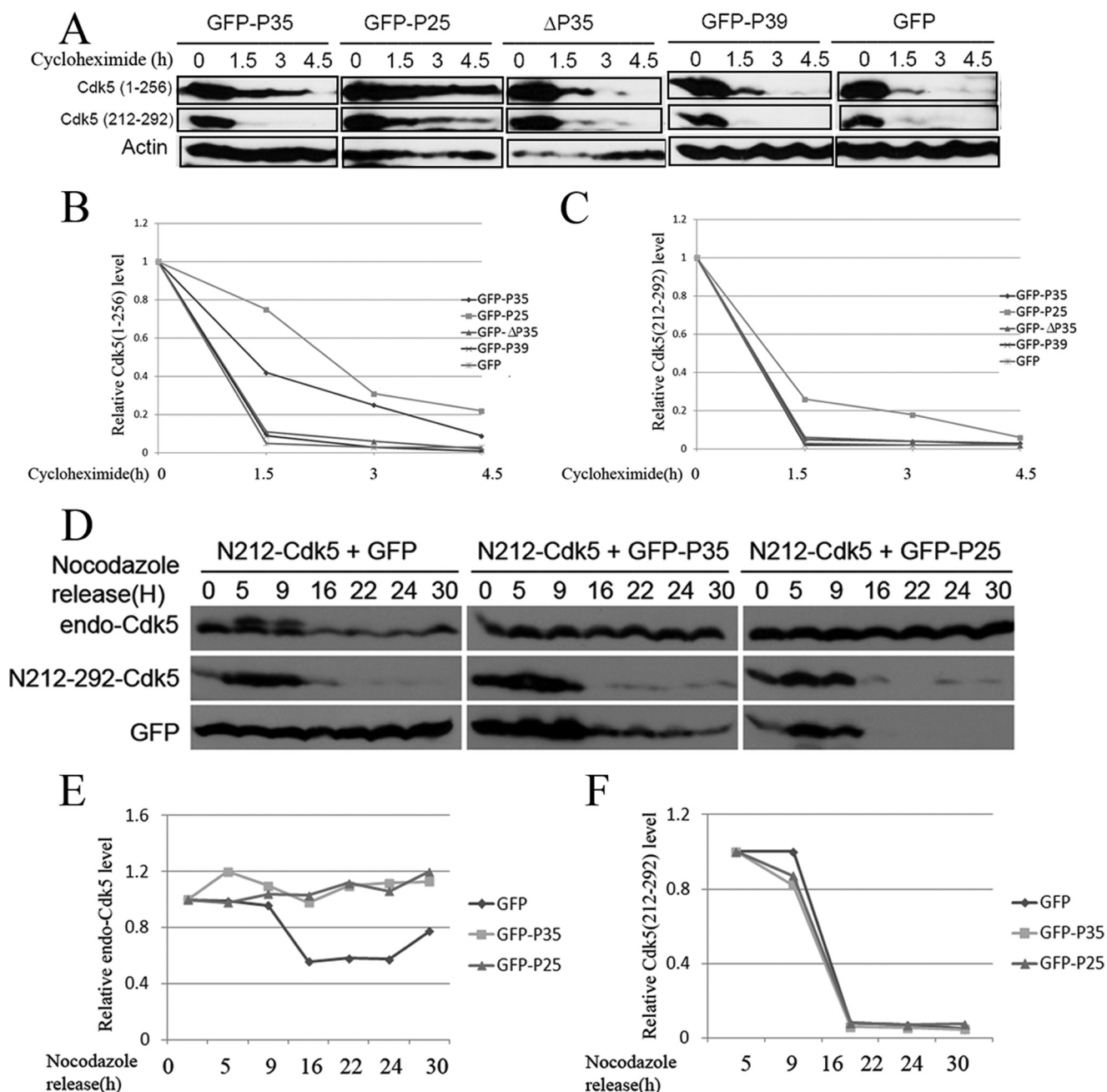
FIGURE 7. **p35 blocks the degradation of Cdk5.** *A–C,* protein stability of Cdk5 was measured after blocking new protein synthesis for varying lengths of time with cycloheximide. N2a cells were co-transfected with either Cdk5(1–256) or Cdk(212–292) plus GFP-tagged p35, p25, Δp35, p39, or GFP alone. After administration of cycloheximide, cells were harvested in 0, 1.5, 3, and 4.5 h. The protein levels were detected by Western blotting; their degradation curves are shown in *panels B* and *C*. Actin serves as a loading control. *D–F,* comparison of exogenous and endogenous Cdk5 during the cell cycle. N2a cells were co-transfected a truncated Cdk5N212 with GFP-tagged p35, GFP-p25, or GFP alone. Cells were then synchronized with nocodazole for 18 h, released and harvested at different time points (see Fig. 1 for Ref.). Western blots of the resulting cell lysates were blotted with Cdk5 or GFP as shown. Note that in the presence of p35 or p25, the S-phase degradation of both endogenous and overexpressed cdk5 was significantly reduced. By contrast, the N212 truncation was unaffected by the presence of either p35 or p25. Their degradation curves were shown in *panels E* and *F*.

that APC^{Cdh1} mediates the degradation of Cdk5 in early S-phase. This represents a previously unrecognized G1/S-phase activity for the ligase and raises the question of how it is regulated. One possibility is that the protein components of the APC^{Cdh1} complex change leading to an altered substrate specificity that favors Cdk5 over more traditional targets. It is also possible that there is a change in the subcellular location of APC/C. Cdk5 mediates cell cycle suppression only if located in the nucleus. In adult neurons, APC/C has a cytoplasmic loca-

tion. Indeed, cytoplasmic APC/C^{Cdh1} and APC/C^{Cdc20} have been shown to engage in reciprocal activities. APC^{Cdh1} functions as an inhibitor of axonal growth and patterning (41) and synaptic control (42), while APC^{Cdc20} plays a role in the suppression of dendrite growth (41, 43). Additional complexity is added by the demonstration that Cdk5 can phosphorylate Cdh1. This reduces APC^{Cdh1} activity and leads to the accumulation of Cyclin B1 in neurons (44), followed by their re-entrance into a lethal cell cycle (45).
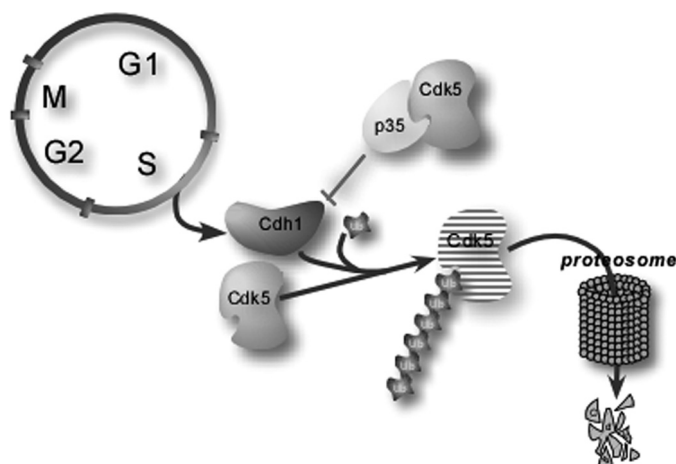
FIGURE 8. **A diagram of our model of the degradation mechanism of Cdk5.** When a neuronal cell enters S-phase, Cdk5 is transported into cytoplasm where it is ubiquitinated by the E3 ligase, APC-Cdh1. The ubiquitinated Cdk5 is then targeted for degradation by the proteasome. Based on the protection data of full-length and truncated Cdk5 constructs, we propose that the ubiquitination site of Cdk5 is in the p35 binding area. Thus, in the presence of p35, the ubiquitination of Cdk5 is blocked, and the degradation of Cdk5 during S phase is attenuated.

This complexity underscores the key role of protein-protein interactions in regulating the levels of Cdk5 protein. Cdk5 binding to either p35 or its p25 breakdown product occludes the Cdh1 ubiquitination site and thus protects Cdk5 from ubiquitin-dependent proteolysis, maintaining its presence in the cell. The importance of p35 binding is demonstrated by the finding that the ΔP35 mutant, which is unable to bind with Cdk5 (18), is unable to block the interaction between Cdk5 and Cdh1 and thus unable to block the ubiquitination of Cdk5. In support of this model, the truncation constructs (Fig. 4) that bind p35 are all protected from ubiquitination and degradation; those that cannot bind p35 are not. As the 4-protein E2F1-p35-Cdk5-p27 complex reported by our laboratory requires p35 to form (20 – 22), we assume that this higher order association also provides protection, but our finding that p25 also protects Cdk5 from ubiquitination and degradation suggests that the other members of the 4-protein complex are not required.

Different cyclins are only capable of activating the kinase activities of specific Cdks. In this context it is noteworthy that, even though p35 can bind to Cdk1–6, it only attenuates the ubiquitination of Cdk5 and Cdk1. This suggests that despite the high degree of structural similarity between p35 and the traditional cyclins, there are Cdk-specific changes that are realized by the p35 protein. The outcome of these interactions is that, in the presence of p35 or p25, the degradation of Cdk5 in S phase is significantly reduced. Fig. 8 summarizes our findings. In either dividing neuronal cell lines, or in mature non-mitotic neurons, the entrance into S-phase is preceded by the translocation of Cdk5 from nucleus to cytoplasm. This is accompanied by the dissociation of Cdk5 from the 4-protein complex and from p35/25, followed by its association with, and ubiquitination by, APC^{Cdh1} leading to its ultimate degradation by the ubiquitin-proteasome pathway. As the Cdk5 kinase has been proposed to play a critical role in the pathogenesis of Alzheimer disease, our data offer new insight into the different ways in which Cdk5 levels can be regulated.

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